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## Inter-kingdom complementation discloses functional convergence of lipocalins

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1 **Inter-kingdom complementation discloses functional convergence**  
2 **of lipocalins.**

3  
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11

12 **Abstract**

13 **Background**

14 The chloroplastic lipocalin (LCNP) is induced in response to various abiotic stresses including  
15 high light, dehydration and low temperature. It contributes to protection against oxidative  
16 damage promoted by harmful conditions by preventing accumulation of hydroxy fatty acids  
17 and lipid peroxidation. In contrast to animal lipocalins, LCNP is poorly characterized and the  
18 molecular mechanism by which it exerts protective effects during oxidative stress is largely  
19 unknown. LCNP is considered the ortholog of human apolipoprotein D (APOD), a protein  
20 whose lipid antioxidant function has been characterized. Here, we investigated whether APOD  
21 could functionally replace LCNP in Arabidopsis.

22 **Results**

23 We introduced APOD cDNA fused to a chloroplast transit peptide encoding sequence in an  
24 Arabidopsis LCNP KO (knock-out) mutant line and challenged the transgenic plants with  
25 different abiotic stresses. We demonstrated that expression of human APOD in Arabidopsis can  
26 partially compensate for the lack of the plastid lipocalin. However, if the results obtained with  
27 the drought and oxidative stresses point to the protective effect of constitutive expression of  
28 APOD in plants lacking LCNP, this effect is not as effective as that conferred by LCNP  
29 overexpression. Moreover, when investigating APOD function in thylakoids after high light  
30 stress at low temperature, it appeared that APOD could not contribute to qH, a slowly reversible  
31 form of non-photochemical chlorophyll fluorescence quenching, as described for LCNP.

## 32 **Conclusions**

33 The results are consistent with a conserved function of APOD and LCNP under stressful  
34 conditions. The work contributes towards understanding the molecular mechanism of action of  
35 LCNP in oxidative stress protection and provides the basis for new research assumptions.

36

37

38 **Keywords: Apolipoprotein D, Lipocalin, Chloroplast, Oxidative stress, Arabidopsis**

39

## 40 **Background.**

41 Lipocalins constitute an evolutionary conserved family of small proteins widely distributed in  
42 nature whose common feature is their ability to bind small hydrophobic molecules [1].  
43 Although their primary amino acid sequences are not highly conserved, they all display a  
44 repeated structurally conserved region and an eight-stranded antiparallel  $\beta$ -sheet that together  
45 form a compact barrel structure with a calyx-shaped ligand pocket [2]. Shape, size and  
46 properties of this pocket vary among family members in line with the different ligands that can  
47 be accommodated. A large variety of ligands including lipids, steroids as well as secondary  
48 metabolites such as vitamins, cofactors and odorants have been reported [1, 3]. Related to their  
49 ligands, lipocalins can fulfill a wide variety of functions such as transport of small molecules,  
50 regulation of developmental processes, signal transduction and response to stress. Because of  
51 their possible involvement in various diseases including lipid disorders, neurodegenerative  
52 diseases and cancer [4, 5], human and animal lipocalins are extensively studied.

53 In striking contrast, plant lipocalins are poorly characterized. The first lipocalins reported in  
54 plants were the violaxanthin-de-epoxidase (VDE) and the zeaxanthin epoxidase (ZEP), two  
55 enzymes involved in the xanthophyll cycle [6]. Given the peculiar molecular and structural  
56 features of both enzymes and the strict definition of lipocalins, VDE and ZEP are now classified  
57 as lipocalin-like proteins [7]. Genomic data mining demonstrated that plants possess also true  
58 lipocalins, which were classified as temperature-induced lipocalin (TIL) and chloroplastic  
59 lipocalin (LCNP<sup>1</sup>) [7]. Investigation of gene regulation, proteomic analyses and reverse genetic  
60 studies have related TIL and LCNP with stress response and tolerance. TIL appeared essential  
61 for thermotolerance [8], its accumulation during cold acclimation was described in Arabidopsis  
62 leaves [9] and in Siberian spruce needles [10]. *TIL* gene expression has been demonstrated to

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<sup>1</sup> Initially named CHL, the chloroplast lipocalin was renamed LCNP ["Lipocalin in the Plastid"] by Malnoë et al. in 2018. This abbreviation will be used throughout the text for designating the plastid lipocalin.

63 increase with heat and cold treatment in wheat and Arabidopsis [11] and peach fruits [12]. In  
64 *Oryza sativa*, a TIL encoding gene was reported to be highly up regulated under heat stress in  
65 panicles of tolerant rice cultivar [13]. *TIL* expression was also significantly induced by a cold  
66 treatment in *Medicago sativa* subsp. *falcata*, a forage legume cold tolerant [14] and in a  
67 grapefruit following a conditioning treatment that enhances chilling tolerance [15]. In grape  
68 berries of *Vitis labruscana*, salicylic acid treatment up-regulated *TIL* preventing postharvest  
69 loss during cold storage [16]. In mango fruits a TIL homolog and its corresponding gene  
70 appeared to be up-regulated during a brassinolide-mediated response to cold stress [17].  
71 Moreover, TIL plays a role in salt tolerance in *Populus euphratica*, a salt-tolerant poplar species  
72 [18, 19].

73 Considering LCNP, it has been demonstrated that high light, dehydration, oxidative stress and  
74 abscisic acid (ABA) induced transcript and protein accumulation in Arabidopsis [20]. In wheat,  
75 cold acclimation induced *LCNP* mainly in freezing-tolerant cultivar while heat exposure down-  
76 regulated gene expression [7]. In *Festuca arundinacea*, salt stress induced a LCNP protein level  
77 increase, which was higher in a salt-tolerant genotype [21]. Furthermore, LCNP abundance  
78 similarly decreased in response to drought treatment in two cultivars of *F arundinacea*,  
79 however, after rewatering lipocalin level returned to initial level only in the high-drought-  
80 tolerant genotype [22].

81 In spite of the accumulating knowledge regarding the contribution of TIL and LCNP to the  
82 resistance of plants to environmental constraints little is known about their role at the cellular  
83 and molecular levels. It has been suggested that plant lipocalins prevent accumulation of  
84 hydroxy fatty acids and thus stop the lipid peroxidation chain reactions initiated by reactive  
85 oxygen species (ROS) generated during stresses [8, 23]. In 2009, Levesque-Tremblay et al. [20]  
86 demonstrated that when exposed to excess light, *LCNP* KO plants display a rapid accumulation  
87 of hydroxy fatty acids relative to the wild-type, whereas the lipid peroxidation level remains

88 very low in *LCNP* overexpressing plants. Boca et al. [24] demonstrated that *LCNP* deficiency  
89 dramatically enhances the photosensitivity of mutants affected in lipid protection mechanisms  
90 strengthening the hypothesis that lipocalins prevent lipid peroxidation. Interestingly, recent data  
91 obtained by Malnoë et al. [25] demonstrated a link between *LCNP* and qH a slowly reversible  
92 form of non-photochemical energy quenching (NPQ), a mechanism by which photosynthetic  
93 organisms harmlessly dissipate excess absorbed light energy. The authors suggested that the  
94 accumulation of peroxidized lipids observed in *Arabidopsis lcnp* mutants following abiotic  
95 stress is a consequence of the absence of the photoprotective NPQ mechanism enabled by the  
96 plastid lipocalin and, thus, that *LCNP* might function in preventing the formation of peroxidized  
97 lipids. In addition to or as part of its role in NPQ, it is suggested that *LCNP* might detoxify  
98 peroxidized lipids directly [25].

99 Phylogenetic studies established that plant lipocalins share similarities with the bacterial  
100 lipocalin, the mammalian apolipoprotein D (APOD) and the insect Lazarillo protein. As observed  
101 in plants, the correlation between lipid oxidation status and lipocalin abundance was reported  
102 for animal and human organisms. For instance in mice, loss of *APOD* increased lipid  
103 peroxidation in the brain [26]. Conversely, human *APOD* overexpression reduced lipid  
104 peroxidation in mice and *Drosophila* [26, 27]. Increased *APOD* levels in the human brain were  
105 reported under conditions that promote lipid peroxidation such as aging or Alzheimer's disease  
106 [28]. At the biochemical level, *APOD* ligands are well-known [29, 30, 31] and the molecular  
107 mechanism underlying the antioxidative properties of *APOD* has been established. Indeed, the  
108 ability for *APOD* to control lipid peroxidation by catalyzing the reduction of radical-  
109 propagating lipid hydroperoxides has been characterized [32].

110 In order to characterize the mechanism underlying the protective role of *LCNP* against abiotic  
111 stresses we investigated whether *APOD* whose biochemical properties involved in oxidative  
112 stress tolerance have been described, is functionally orthologous to the chloroplast lipocalin.

113 To do so, human APOD cDNA fused to a chloroplast transit peptide encoding sequence was  
114 introduced in an Arabidopsis *LCNP* KO mutant line. Transgenic plants expressing *APOD* were  
115 challenged with different abiotic stresses. The plants displayed partial tolerant phenotype  
116 suggesting that human APOD can replace in part, *LCNP*. These data provide a base of  
117 understanding the antioxidative mechanism of the plastid lipocalin and some testable  
118 experimental assumptions.

119

## 120 **Results**

### 121 *Targeting the human APOD to Arabidopsis thaliana chloroplast: gene expression and* 122 *protein localization.*

123 To determine whether *APOD* is functionally orthologous to *LCNP* we took an *in vivo*  
124 complementary approach expressing the human APOD encoding sequence in Arabidopsis. In  
125 order to target APOD to the chloroplast where *LCNP* is located, the putative transit peptide  
126 encoding sequence of *LCNP* was fused to the cDNA coding for APOD (Fig. 1A). The chimeric  
127 construct under the control of the Cauliflower Mosaic virus (CaMV) 35S promoter was  
128 introduced in an Arabidopsis mutant line lacking *LCNP* [20]. Primary transgenic Arabidopsis  
129 plants were identified following Basta treatment of one-week old seedlings. In the T2  
130 generation, 5 independent lines containing a single insertion were selected by analyzing the  
131 segregation ratio of herbicide resistance. Seeds were collected from homozygous T3 plants and  
132 used for further experiments.

133 The 5 transformant lines a661, a351, a421, a551, and a212 showed no phenotypic changes  
134 compared to wild type. In the five transgenic lines, *APOD* expression was analyzed by reverse  
135 transcriptase polymerase chain reaction (RT-PCR, Fig. 1B). Two lines a661 and a421 exhibited  
136 a higher expression as compared to the three other lines. At the protein level, immunoblot

137 analysis was conducted to detect the presence of APOD (Fig. 1C). A strong band was observed  
138 in the two transgenic lines containing the higher amount of *APOD* transcripts (a661 and a221)  
139 while the protein was hardly detectable in the three other lines. The molecular weight of the  
140 band was estimated at 23 KDa, which does not correspond to the molecular weight of APOD  
141 estimated at 19.3 KDa suggesting either that the transit peptide was not properly processed or  
142 that APOD was post-translationally modified.

143 Within chloroplasts, LCNP was localized in the thylakoid lumen using a cellular fractionation  
144 approach [20]. To investigate APOD subcellular localization we performed western-blot  
145 experiments on plastid sub-fractions isolated from chloroplasts purified by Percoll gradient  
146 centrifugation (Fig. 2). APOD appeared to be of low abundance in the total cell extract. As  
147 expected its concentration slightly increased in purified chloroplasts where the human lipocalin  
148 was present in both compartments, thylakoids and stroma. However, in NaCl stripped  
149 thylakoids APOD could not be detected suggesting that the protein is ionically attached to the  
150 membranes. These results suggest that in chloroplasts, APOD remains soluble in the stroma  
151 and also ionically attaches to the thylakoids.

152 To investigate further the localization of APOD in transgenic plants, the protein was tagged  
153 with EGFP at its C-terminus and transiently expressed using the CaMV 35S promoter in  
154 *Nicotiana benthamiana* leaves (Fig. 3). Six days later, leaf epidermal cells were observed by  
155 confocal microscopy. EGFP fluorescence coincided strongly with chlorophyll autofluorescence  
156 (Fig. 3A) indicating that, as expected, APOD:EGFP fusion product was associated with the  
157 chloroplast. In particular, confocal imaging revealed a strong fluorescence within the guard cell  
158 chloroplasts (Fig. 3A). Furthermore, EGFP fluorescence was also detected in other subcellular  
159 structures throughout the cell. To identify EGFP labeled structures co-localization experiments  
160 were performed with different compartment markers (Fig. 3B and 3C). Fluorescence was  
161 evident in the endoplasmic reticulum network (ER) and in Golgi as demonstrated by the co-



162 localization of the tagged protein with the mCherry signal targeted either to the ER (Fig. 3B)  
163 or to the Golgi (Fig. 3C). In contrast co-localization experiments performed with a marker of  
164 plasmalemma and tonoplast excluded the presence of proteins in these membranes (data not  
165 shown).

### 166 ***Effect of APOD expression on abiotic stress tolerance***

167 The chloroplast lipocalin plays an important role in tolerance to environmental constraints. In  
168 Arabidopsis it has been demonstrated that a high level of LCNP helped plants to cope with  
169 environmental abiotic stresses such as drought, high light, cold acclimation and oxidative stress.  
170 Conversely plants lacking LCNP exhibited more damage under abiotic stresses [20]. In order  
171 to investigate whether APOD could also confer stress tolerance and play a role similar to that  
172 of LCNP in protecting plants, the transgenic lines (a661 and a421) expressing *APOD* in the  
173 *LCNP* KO background were exposed to different stress conditions including drought (Fig. 4)  
174 and oxidative stress (Fig. 5).

175 Arabidopsis *lcnp*, wild-type and transgenic seeds were planted in a mix of soil and sand. Three  
176 weeks later, water was withheld for 10 days. *lcnp* mutants showed severe withering symptoms  
177 as compared to wild type plants, attesting of the great sensitivity of these plants to a water  
178 deficit. The plants over-expressing *LCNP* (*LCNP O/E*) and those expressing *APOD* were only  
179 slightly wilted (Fig. 4A). When plants were re-watered, none of the KO mutant plants survived  
180 (Fig. 4B). In contrast, Col0, *LCNP O/E* and the two lines containing *APOD* coped well with  
181 the stress conditions. These results suggested that *APOD* expression in the drought-sensitive  
182 *lcnp* genotype could restore tolerance to a mild water stress to the level recorded in wild type  
183 plants.

184 Previous studies indicated that LCNP is involved in the protection of the photosynthetic  
185 apparatus against ROS. Therefore we have tested resistance of plants against cellular oxidative  
186 damage by applying the herbicide paraquat (PQ, methyl viologen) to leaf disks segments from

187 transgenic plants. The electrons produced during photosynthesis reduce PQ and free oxygen  
188 radicals are formed initiating an oxidative stress and quickly damaging the photosynthetic  
189 apparatus. Leaf disks were exposed to 1  $\mu$ M PQ for 8 hours under a light source. Functional  
190 damage was monitored by measuring the variable fluorescence (Fv)/maximal fluorescence  
191 (Fm) values representing the maximal yield of photochemistry in the photosynthetic process  
192 (Fig. 5). The control and *lcnp* mutant completely lost their photosynthetic function. As expected  
193 the *LCNP O/E* line was significantly more resistant to PQ treatment. Interestingly, plants  
194 expressing *APOD* (a661 and a421) exhibited a moderate resistance while a212, a line exhibiting  
195 a very low expression of *APOD* displayed an oxidative stress-sensitive phenotype similar to the  
196 control (Col0). Considering these results we can conclude that the constitutive presence of  
197 *APOD* confers enhanced tolerance to PQ-induced oxidative stress. However oxidative  
198 tolerance did not appear as notable as that recorded in *LCNP* overexpressing plants.

#### 199 ***Effect of APOD expression on sustained photoprotective energy dissipation***

200 It has been demonstrated that *LCNP* contributed to qH, a NPQ component, which operates  
201 under stress conditions such as cold and high light and appeared to be photoprotective [25].  
202 Whether *APOD* can replace *LCNP* in this function was investigated. Detached leaves of  
203 transgenic plants were exposed to high light at low temperature for 6 hours and maximal  
204 fluorescence Fm was recorded. The *suppressor of quenching 1* mutant (*soq1*) with enhanced  
205 qH whose phenotype is reversed when *LCNP* is lacking was included in the experiment. Before  
206 the stress, Col0 and the different mutant and transgenic lines displayed similar maximal  
207 fluorescence parameter (Fm, data not shown). After stress (Fig. 6), as expected since already  
208 described by Malnoë et al. [25], *soq1* displayed a lower Fm compared with Col0 while Fm in  
209 *lcnp* was higher than in the control plant leaves. The photoprotective function of *LCNP* was  
210 observed in the leaves of the transgenic line overexpressing *LCNP* with a maximal chlorophyll  
211 fluorescence parameter lower than in Col0. In comparison, a661 and a421 displayed a Fm

212 similar to that recorded in *lcnp* indicating that *APOD* expression cannot protect the thylakoid  
213 membrane as LCNP does.

## 214 **Discussion**

215 Discussion

216 We report that expression of human APOD in Arabidopsis, by means of transgenic lines, can  
217 partially compensate for the lack of the plant lipocalin, LCNP. The results are consistent with  
218 a conserved function of APOD and LCNP under stressful conditions. However, if the results  
219 obtained with the drought and oxidative stresses point to the protective effect of constitutive  
220 expression of APOD in plants lacking LCNP, this effect is not as effective as that conferred by  
221 LCNP overexpression. Moreover, when investigating APOD function in NPQ after high light  
222 stress at low temperature, it appeared that APOD could not contribute to qH as described for  
223 LCNP [25]. It is intriguing to notice that in Arabidopsis, APOD can fulfill only certain functions  
224 of LCNP.

225 Molecular phylogenetic analysis of lipocalins not only revealed that the chloroplastic LCNP  
226 shared significant structural homology with the mammalian APOD [7, 33], but also suggested  
227 that both proteins might correspond to the same overall function in terms of general nature of  
228 binding ligands since both proteins appeared in the same clade [7, 34]. In vitro ligand binding  
229 assays have shown that APOD is able to bind a variety of lipids in its hydrophobic binding  
230 cavity [29]. More recently, it has been clearly demonstrated that APOD displayed the highest  
231 binding affinity for arachidonic acid [AA] and progesterone [29, 30, 31]. Partial functional  
232 complementation of LCNP deficiency by APOD cannot be related to the nature of APOD  
233 ligands since neither AA nor progesterone are present in Arabidopsis. However we cannot  
234 exclude the possible affinity of APOD to other structurally related lipids that may be involved  
235 in signaling cascades. Indeed numerous signaling lipids whose synthesis can rapidly be  
236 activated upon abiotic stress signals have been identified in plants [for a review see 36]. In

237 Arabidopsis, APOD could exert a control of the signaling lipid molecules that decipher the  
238 stress response cascade and thus modulate transgenic plants tolerance. In plants, a similar  
239 function for LCNP cannot be excluded as well.

240 During last decade, several authors have established that APOD function extended well beyond  
241 the transport of lipophilic molecules. Therefore, an extensive work allowed to establish a  
242 correlation between lipid oxidation status and APOD abundance in human and mammals.  
243 Increased APOD expression was reported in the human brain under conditions that promote  
244 lipid peroxidation and up-regulation of antioxidant gene expression such as aging or  
245 neuropathological conditions including schizophrenia, Parkinson's and Alzheimer's diseases  
246 [28, 35, 37]. Studies in mice showed that loss of APOD increased lipid peroxidation in the  
247 brain, that cerebral APOD expression is increased in response to Paraquat-induced oxidative  
248 stress and that human APOD expression reduced lipid peroxidation [26, 27]. In addition,  
249 expression of human APOD reduced the accumulation of aldehydic end-products of lipid  
250 peroxidation in an aged *Drosophila* model [27, 38]. In plants, the protective role of LCNP in  
251 oxidative stress has been related to its ability to prevent or modulate lipid peroxidation caused  
252 by ROS action [20]. Thus, a LCNP KO Arabidopsis line, which exhibited serious damage upon  
253 photo-oxidative stress induced by drought, high light or paraquat displayed a rapid  
254 accumulation of hydroperoxyl fatty acids. In contrast, in LCNP over-expressing lines, which  
255 coped better with these conditions, the lipid peroxidation level remains very low [20]. Related  
256 to APOD antioxidative function, it has been demonstrated that the human lipocalin had the  
257 capacities to scavenge hydroxyl radicals [40] and catalyze the reduction of hydroperoxide of  
258 AA to the non-reactive alcohols, thereby preventing lipid peroxidation chain reactions [32].  
259 This reaction involves a highly conserved methionine residue (Met93 in human APOD) located  
260 at the hydrophobic surface of the protein [32] and results in oxidation of the methionine  
261 sulfhydryl to form methionine sulfoxide (MetSO). A similar molecular function could be

262 involved to explain the protective role of APOD in Arabidopsis. Indeed, it can be speculated  
263 that APOD could exert its reductive activity on plant lipid hydroperoxides such as  
264 hydroperoxides of linolenic (18:3), linoleic (18:2) and roushanic (16:3) acids, the major  
265 polyunsaturated fatty acids in plants. In this context, APOD could inhibit lipid peroxidation  
266 propagation initiated by drought and paraquat treatment. A lower affinity of APOD for plant  
267 hydroperoxides or an insufficient amount of APOD in plant cells could account for the lower  
268 protective effect of the human lipocalin as compared to that provided by LCNP. By analogy,  
269 the protective function towards oxidative stresses conferred by LCNP could be due to a surface  
270 methionine residue, similar to APOD Met93 and able to reduce plant fatty acid hydroperoxides,  
271 as well. In that respect, we investigated whether LCNP could exhibit a methionine residue  
272 structurally homolog of APOD Met93. In the absence of an experimentally-determined protein  
273 structure, homology modeling using Phyre2 provided a three-dimensional structure for LCNP.  
274 Structural alignment with the human APOD model is shown in Fig 7. The APOD Met93 is not  
275 conserved in LCNP ruling out the contribution of the Met93 homolog in LCNP oxidative  
276 protective function. However, LCNP contains many other Met residues whose putative  
277 reductive activity should be investigated in future studies [7].

278 The appropriate subcellular localization and translocation of lipocalins are crucial to their  
279 functionality. LCNP located into the lumen of thylakoids [20] has been shown to be involved  
280 in qH, which has been localized to the peripheral antenna of photosystem II, suggesting that  
281 LCNP could either directly form a NPQ site or indirectly modify the light harvesting complex  
282 II (LHCII) membrane environment [25]. Lumen targeting is a complex process, which may  
283 involve two different pathways able to recognize a thylakoid-targeting signal peptide [41]. The  
284 N-terminal bipartite signal for chloroplast import and thylakoid transfer that drives the import  
285 of LCNP from the cytosol to the lumen has not yet been characterized. In our construct, LCNP  
286 transit peptide extension was defined based on unpublished data [Malnoë, personal

287 communication]. In Arabidopsis transgenic plants, APOD fused to the putative transit peptide  
288 of LCNP was clearly targeted to the chloroplast (Fig. 2 and Fig. 3). However we could not find  
289 evidence that APOD penetrated into the lumen of thylakoids. Inappropriate processing could  
290 explain the APOD unexpected size described in Fig. 1 and the lack of APOD in the lumen.

291 APOD is a glycoprotein and we cannot exclude that glycosylation occurred in cells of  
292 transgenic plants. The localization of APOD fused to GFP into Golgi and ER supports this  
293 hypothesis (Fig. 3). Moreover, this post-translational modification could explain the  
294 unexpected molecular weight of APOD in a plant cell extract (Fig. 2). Lastly, targeting of  
295 glycoproteins into chloroplasts is well known [42] but import into thylakoids lumen has never  
296 been described. The lack of lipocalin into the chloroplast sub compartment, although the protein  
297 is present in the stroma, may explain why APOD did not contribute to qH as LCNP does.

## 298 Conclusions

299 Our data indicate that despite structural homology and similar contribution to lipid oxidation  
300 status in cells, APOD and LCNP display molecular features that confer specific functions to  
301 them. We propose that functionality and specificity likely reside in a particular methionine  
302 residue and glycosylation modifications that affect enzyme activity and subcellular localization,  
303 respectively. The findings of this study provide new leads for future research. In particular, we  
304 are examining LCNP methionine residues to assess their contribution in lipid hydroperoxide  
305 reduction.

306

## 307 **Methods**

### 308 *Plant Material, Growth Conditions and Treatments*

309 Arabidopsis plants were grown in phytotrons with an 8/16 day/night photoperiod and a light  
310 intensity of  $130 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Day and night temperatures were 22 and 18 °C, respectively. The

311 relative humidity was set at 60 % and plants were cultivated at 100% relative soil water content.  
312 For drought stress, 9 plants of each line were grown in a single pot containing 120 g of a mix  
313 of soil and sand (2/1, w/w). Three week old plants were then subjected to water deprivation by  
314 withholding watering for 10 days. For the oxidative stress treatment, about 9 mm diameter leaf  
315 disks were excised from fully expanded leaf blades and floated onto a 1  $\mu$ M solution of paraquat  
316 (PQ, N,N'-dimethyl-4,4'-bipyridinium dichloride, Sigma-Aldrich) dissolved in water for 8  
317 hours.

318 *Nicotiana benthamiana* plants were grown in soil under controlled conditions in a growth  
319 chamber (14-h photoperiod at 25°C followed by a 10-h night at 20°C). Arabidopsis and *N*  
320 *benthamiana* plants were watered with a complete nutrient solution as previously described  
321 [43].

322 The *lcnp* KO mutant line (*lcnp-1*) was available through the European Arabidopsis stock Center  
323 at Nottingham University. The *LCNP* overexpressing line was provided by Dr F Ouellet from  
324 Université du Québec (Montréal, Canada).

### 325 ***Chlorophyll Fluorescence Measurements***

326 Chlorophyll fluorescence from leaf disks was measured at room temperature using a PAM-  
327 2000 modulated fluorometer (Walz, <http://www.walz.com>) as previously described [25]. The  
328 maximal quantum yield of photosystem II photochemistry was measured as

$$329 \quad F_v/F_m = [F_m - F_0] / F_m$$

330 where  $F_m$  is the maximal fluorescence level (measured using a 800 msec pulse of saturating  
331 light) and  $F_0$  is the initial fluorescence level (measured after a 2 sec pulse of far-red light).

332 For the comparison of the different experimental groups, statistical differences of means were  
333 analyzed with the Student's t or ANOVA tests. Letters have been used to mark statistically  
334 identical groups of means. P values are given in the legend of figure for each group of means..

335 Chlorophyll fluorescence imaging was done with a laboratory-built instrument as described by  
336 Johnson et al. [44].

### 337 ***RT-PCR***

338 Total RNA extraction, cDNA preparation, and RT-PCR were performed as previously  
339 described [45]. Expression of *APOD* was analyzed by RT-PCR with specific primers TepGW  
340 and GWapoD (Additional file 1). As control, RT-PCR was performed using actin gene- specific  
341 primers (At2g37620, Additional file 1).

### 342 ***Construction of Transgenic Plants***

343 DNA sequences, which encode human APOD and the putative transit peptide of LCNP from  
344 Arabidopsis (At3g47860) were amplified by polymerase chain reaction (PCR). The primers  
345 used for PCR are indicated in Fig. 1A, their sequences are listed in Additional file 1. The final  
346 PCR product displayed *attB* recombination sites, which allowed cloning in pDONR201 vector  
347 using a Gateway BP Clonase enzyme mix (Invitrogen). One entry clone was fully sequenced  
348 before subsequent cloning in the binary Gateway destination vectors pB2GW7 and pB7FWG2  
349 (Plant Systems Biology, VIB-Ghent University, Belgium; [46]) by using a Gateway LR  
350 Clonase enzyme mix (Invitrogen). The pB2GW7 vector allows expression of the cloned  
351 sequence and pB7FWG2 vector allows fusion of EGFP with the APOD C-terminus. For both  
352 vectors, the cDNA is placed under the control of the 35S CaMV promoter and of the 3'  
353 untranslated transcribed region of a nopaline synthase gene. The binary constructs were  
354 introduced into the *Agrobacterium tumefaciens* strain C58. The resulting bacterial culture  
355 containing the recombinant pB2GW7 was used to transform Arabidopsis ecotype Columbia-0  
356 (Col0) the standard flower dip method [47]. Transgenic plants were selected in soil after Basta  
357 treatment. The recombinant *Agrobacterium* strain containing the pB7FWG2 construct was  
358 infiltrated in *Nicotiana benthamiana* leaves according to the protocol described by Leuzinger  
359 et al. [48].



### 360 ***Chloroplast Purification and Chlorophyll Measurement***

361 Intact chloroplasts from Arabidopsis leaves were extracted and purified by isopycnic  
362 centrifugation according to Seigneurin-Berny et al. [49]. All operations were carried at 5°C. In  
363 brief, Arabidopsis leaves (100 g, from 5 week-old plants) were ground in 400 mL of grinding  
364 medium (20 mM, Tricine, pH 8.4 containing 0.4 M, Sorbitol; 10 mM, Na<sub>2</sub>-EDTA; 10 mM,  
365 NaHCO<sub>3</sub>; 0.1% [w/v] Bovine serum albumin) using a Waring blender. Chloroplasts were  
366 collected by low speed centrifugation at 2070 x g for 2 min. The chloroplasts in the pellet were  
367 re-suspended in washing buffer (20 mM, Tricine, pH 7.6; 0.4 M, Sorbitol; 2.5 mM, MgCl<sub>2</sub>;  
368 1.25 mM, Na<sub>2</sub>-EDTA) and loaded on the top of a preformed continuous Percoll gradient. Intact  
369 chloroplasts, which concentrated in a green band at the lower part of the gradient were  
370 recovered and rinsed with washing buffer. The upper part of the gradient contains broken  
371 chloroplasts. Following low speed centrifugation (2070 x g for 2 min), chloroplasts were  
372 osmotically lysed in 10 mM Tris-HCl, pH 8. The thylakoid membranes were collected by  
373 centrifugation. Stromal proteins, which remained in the supernatant were acetone precipitated.  
374 Thylakoids were resuspended in hypotonic buffer containing 200 mM NaCl and finally  
375 concentrated by low speed centrifugation.

376 Chlorophyll was extracted with 80 % [v/v] aqueous acetone and quantified by  
377 spectrophotometry following the Arnon's method.

### 378 ***Protein Extraction, SDS-PAGE and Western Blotting***

379 Preparation of a total protein extract from Arabidopsis leaves was performed according to  
380 Rumeau et al. [42]. Following grinding in liquid nitrogen, the powder was resuspended in  
381 extraction buffer [10 mM Tris-HCl pH 8 containing 20 mM NaCl, 2 mM Na<sub>2</sub>-EDTA, 5 mM  
382 Dithiothreitol]. The extract was filtrated trough two layers of muslin and the proteins were  
383 acetone [80 %; v/v] precipitated. Denaturing SDS-PAGE was performed as described by  
384 Laemmli [45] using 13 % acrylamide gels. Proteins were either stained with BioSafe Coomassie

385 (BioRad) or electro transferred onto 0.45  $\mu\text{m}$  nitrocellulose membrane (Biotrace Pall) and  
386 probed with antibodies. Antibodies against APOD were purchased at Sigma-Aldrich/Merck.  
387 Immunocomplexes were visualized with 1:5000 diluted anti-rabbit IRDye800 antibody  
388 (Invitrogen). Detection was enabled by the Odyssey infrared imaging system.

### 389 ***Confocal Microscopy Observation***

390 Pieces of *N benthamiana* leaves were sampled and mounted in water. Imaging was performed  
391 using a Zeiss LSM 780 confocal laser scanning microscope and either a 20x (Plan-Apo) or a  
392 40x (C-Apo Corr FCS) objective. For EGFP and mCherry fluorescence analysis, the 488 nm  
393 excitation line of an argon laser was used and the fluorescence signal was detected using an  
394 emission band width of 490-530 for EGFP and of 590-620 for mCherry.

### 395 ***3D Structure Prediction and Structural Alignment***

396 Three-dimensional structure of LCNP was predicted and modeled from Phyre<sup>2</sup> database [51].  
397 The top model with 100 % confidence was considered. Structural alignment with the human  
398 APOD model (PDB: 2HZR; [30]) was achieved using the Matchmaker tool in the UCSF  
399 Chimera package [52].

400

### 401 **Abbreviations**

402 **ABA:** Abscisic acid; **APOD:** Apolipoprotein D; **CaMV:** Cauliflower Mosaic Virus; **EGFP:**  
403 Enhanced Green Fluorescent Protein; **ER:** Endoplasmic Reticulum; **Fv:** Variable fluorescence;  
404 **Fm:** Maximal Fluorescence; **KO:** Knock-Out; **LCNP:** Plastid Lipocalin; **LHCII:** Light  
405 Harvesting Complex II; **MetSO:** Methionine Sulfoxide; **NPQ:** non-photochemical quenching;  
406 **O/E:** Over Expression; **PAGE:** Polyacrylamide gel electrophoresis; **PCR:** polymerase chain  
407 reaction; **PQ:** Paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride); **PSII:** Photosystem II;  
408 **ROS:** Reactive Oxygen Species; **RT-PCR:** Reverse Transcription Polymerase Chain Reaction;

409 **SDS:** Sodium dodecyl sulfate; **TIL:** Temperature-Induced Lipocalin; **VDE:** Violaxanthin-de-  
410 Epoxidase; **ZEP:** Zeaxanthin Epoxidase

411

## 412 **Declarations**

### 413 *Funding*

414 No funding was obtained for this study.

### 415 *Ethics approval and consent to participate*

416 Not applicable

### 417 *Consent for publication*

418 Not applicable

### 419 *Availability of data and material*

420 Not applicable

### 421 *Competing interests*

422 The authors declare that the research was conducted in the absence of any commercial or  
423 financial relationships that could be construed as a potential conflict of interest.

### 424 *Authors' contributions*

425 DR conceived the project and designed the experiments. DR and PH performed the  
426 experiments. DR wrote the manuscript and PH read and approved it.

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440 Montillet (CEA Cadarache) for critical reading of this manuscript.

441

442

443 **Figure Legends**

444 **Figure 1. Molecular characterization of transgenic plants expressing a chimeric gene**  
445 **containing the LCNP putative transit peptide fused to APOD cDNA.** **A,** The gene structure.  
446 Shaded and white box represent the sequences coding for APOD and LCNP transit peptide  
447 (TP), respectively. Small arrows represent PCR primers whose sequences are listed in  
448 Additional file 1. In the lower part, amino acid sequence of the chimeric protein is also  
449 indicated. In bold, LCNP transit peptide. **B,** RT-PCR analysis of transgenic plants. Total RNA  
450 was extracted from leaves and PCR was performed using TPepGW and GWapoD as gene-  
451 specific primers for the chimeric gene (left panel) and actin gene as an internal control [right  
452 panel]. **C,** Western blot analysis. Total proteins were extracted from leaves and subjected to gel  
453 blot analysis using anti-APOD antibodies. Inset, Coomassie blue stained gel as a loading  
454 control.

455

456 **Figure 2. Subcellular localization of APOD.** Intact chloroplasts were isolated from leaves of  
457 the a661 transgenic line by centrifugation through a Percoll gradient. Chloroplasts were lysed  
458 by hypo osmotic shock and further separated into thylakoid and stroma fractions for  
459 immunoblot analysis. Thylakoids were washed with a buffer containing 200mM NaCl. Proteins  
460 collected following acetone precipitation were separated on a 13% SDS-PAGE. **A,** Coomassie  
461 Blue stained gel; **B,** immunoblot detection of APOD in the chloroplast sub fractions.

462

463 **Figure 3. Co-localization of APOD-EGFP with compartmental markers.** *Nicotiana*  
464 *benthamiana* leaves were agroinfiltrated for the transient expression of APOD:EGFP. Confocal  
465 scanning microscopy observations were performed at 6 days post infiltration. **A,** Chloroplast  
466 marker chlorophyll autofluorescence; **B,** Endoplasmic reticulum marker m Cherry-CNX1; **C,**

467 Golgi marker GNT1-mCherry. (s, stomata). *N benthamiana* infiltrations with the different  
468 constructs were reproduced several times and gave similar results.

469

470 **Figure 4. Tolerance of *Arabidopsis* APOD-containing transgenic lines to water deficit stress.**

471 Nine plants of each line were grown in a single pot containing a mix of soil and sand (2/1, w/w)  
472 under normal conditions for 3 weeks. Then watering was completely withdrawn for 10 days  
473 [A]. Plants were then re-watered for 5 days [B]. These experiments were repeated twice and  
474 gave similar results.

475

476 **Figure 5. Tolerance of *Arabidopsis* APOD-containing transgenic lines to oxidative**

477 **treatment.** Leaf disks were floated on a solution of paraquat [PQ, 1  $\mu$ M] for 8 hours and exposed  
478 to light (130  $\mu$ mol  $m^{-2} s^{-1}$ ). The photosystem II photosynthetic efficiency in the leaf disks was  
479 estimated by chlorophyll fluorescence determination of photochemical yield (Fv/Fm). The data  
480 represent the means  $\pm$  SD (n>6). Student's t- and ANOVA-tests performed, enabled us to  
481 identify 4 groups of statistically different means ( $P \leq 0.001$ ). P values of "t" tests of groups b,  
482 c and d were 0.560, 0.977 and 0.559, respectively.

483

484 **Figure 6. Fluorescence phenotype in APOD-containing transgenic lines induced by cold and**

485 **high light treatment.** Isolated leaves were exposed to cold (6°C) and high-light (1500  $\mu$ mol  
486 photons  $m^{-2} sec^{-1}$ ) treatment for 3 hours. Representative image of maximum chlorophyll  
487 fluorescence emission (Fm) monitored with a fluorescence imaging system.

488

489 **Figure 7. Overlay of the protein structures of human ApoD (purple ribbon) and the homology**  
490 **model of LCNP (blue ribbon).** The methionine residues in both molecules are highlighted in  
491 black. The backbone rmsd is 1,117Å between the mean structures indicating that the global  
492 folds are similar for both proteins despite a low sequence identity (31% identity, 100%  
493 confidence, data not shown).

494

495

496 **Additional file 1.** Sequences of PCR primers designed for the cloning of chimeric genes and  
497 transgenic plants characterization.

498 TepGW

499 GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATATTATTAAGTAGTAGTATA

500 AGC

501 TpepTil2 AACTAGGGAGTGCTGCTGCTACAGAGAAGAAAGAGATGGAA

502 TilPep2 TTCCATCTCTTTCTTCTCTGTAGCAGCAGCACTCCCTAGTT

503 GWtilHA:GGGGACCACTTTGTACAAGAAAGCTGGGTATTAAGCGTAATCTGGAAC

504 ATCGTATGGGTATTTGCCGAAGAGAGATTTGAACCACC

505 Tpep long apoD AACTAGGGAGTGCTGCTGCTCAAGCATTTCATCTTGGGAA

506 apoD Tpep long TTCCAAGATGAAATGCTTGAGCAGCAGCACTCCCTAGTT

507 GWapoD

508 GGGGACCACTTTGTACAAGAAAGCTGGGTATTACGAGAGCTTGGGGCAGTTCAC

509

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651

652



Figure 2

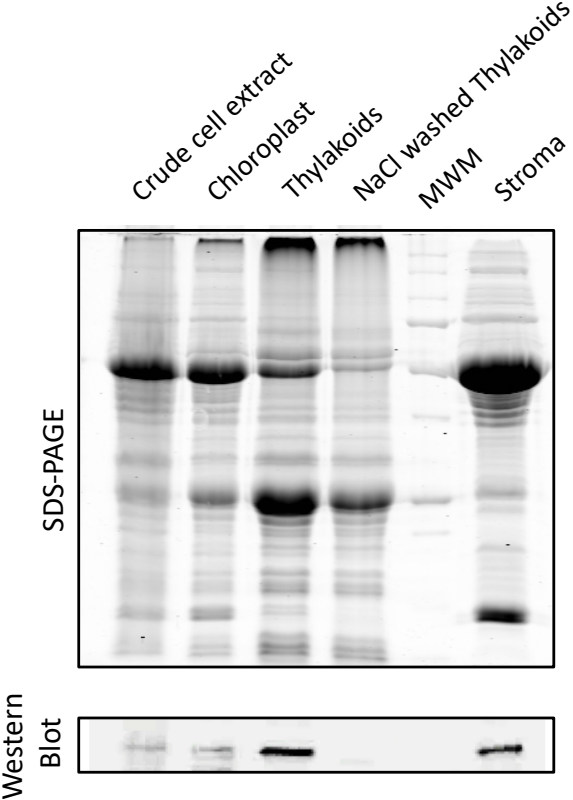
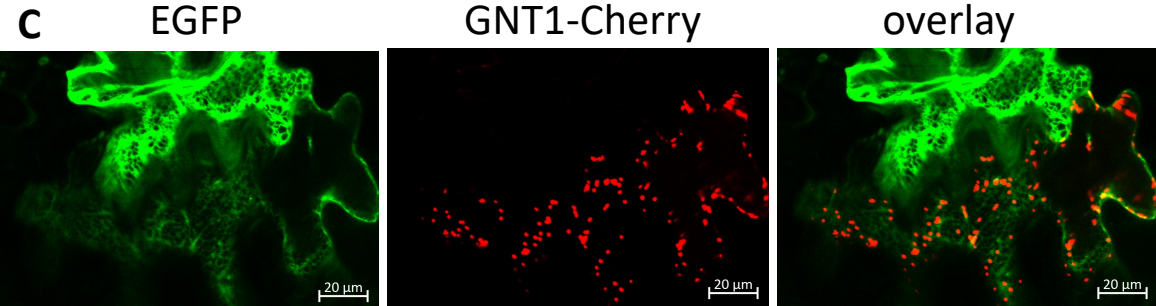
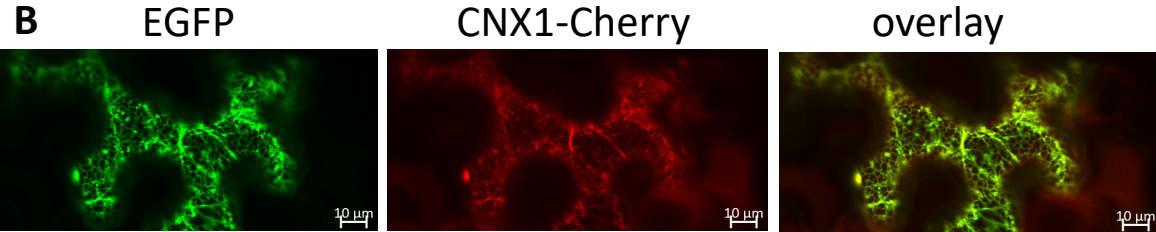
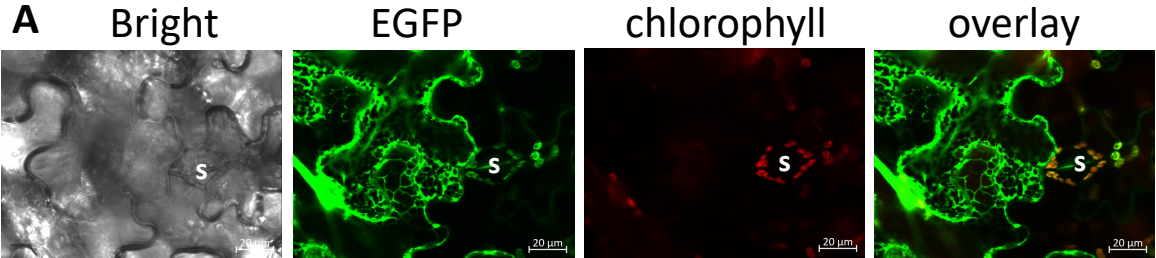


Figure 3



655

656



Figure 4

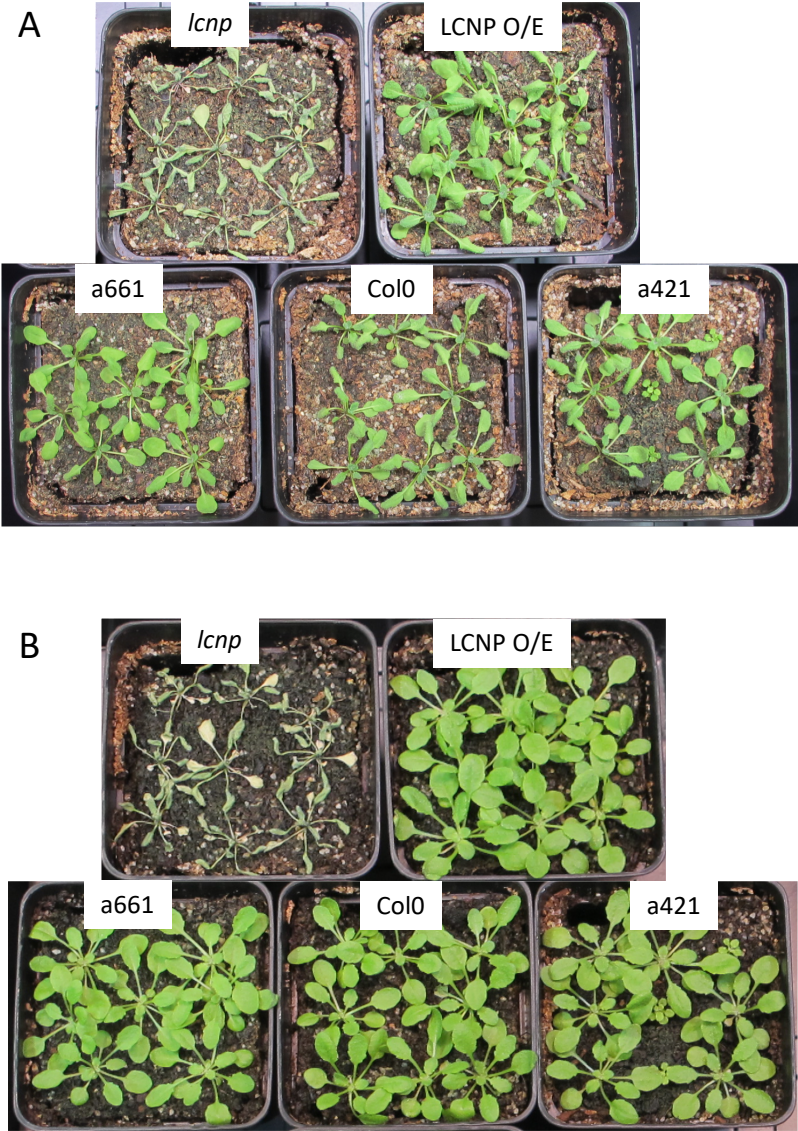


Figure 5

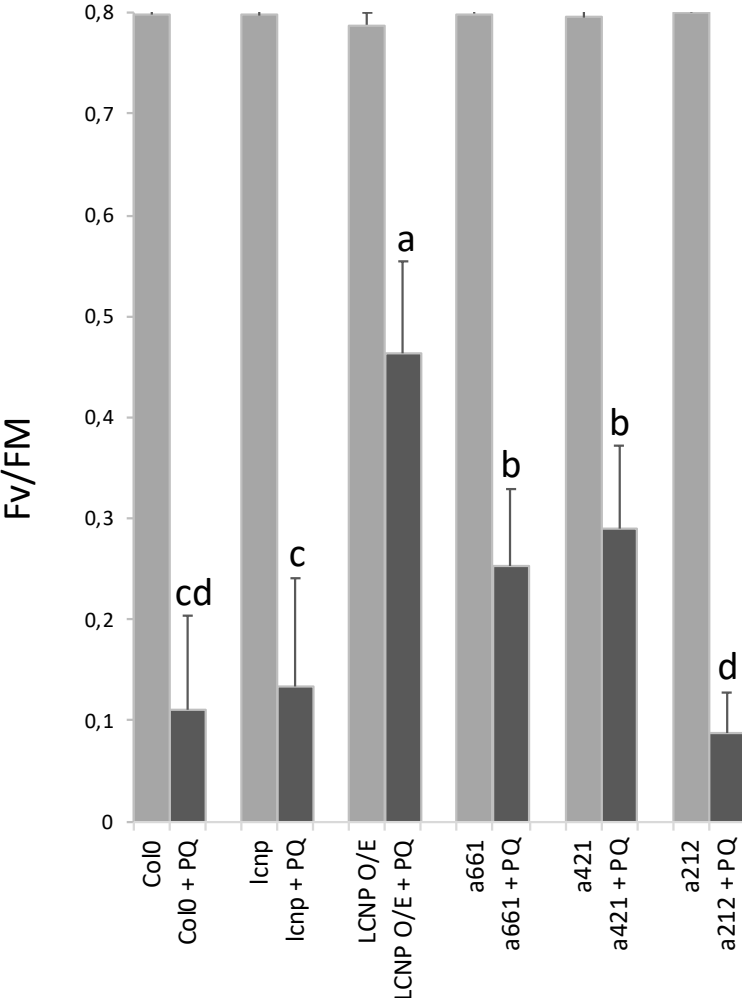


Figure 6

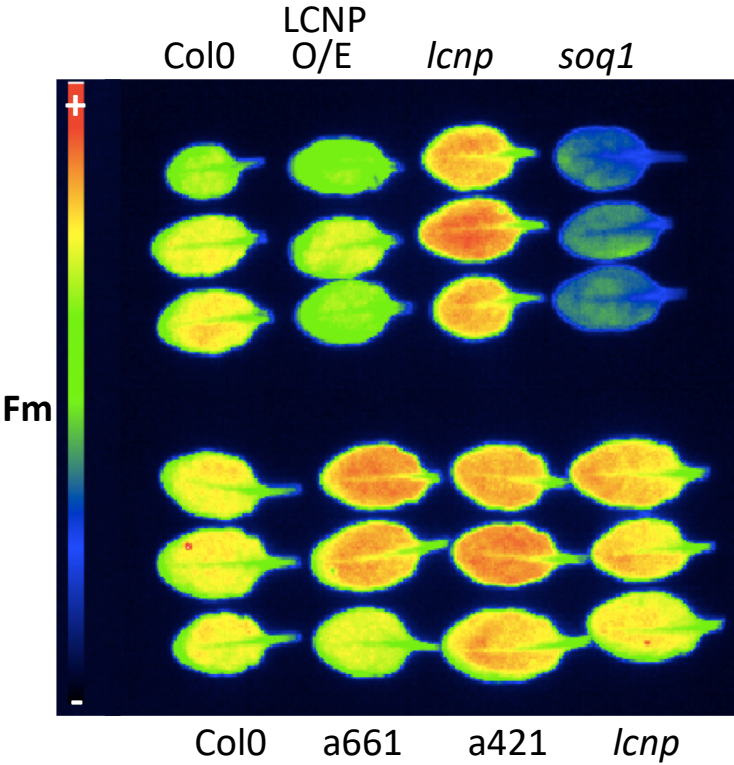
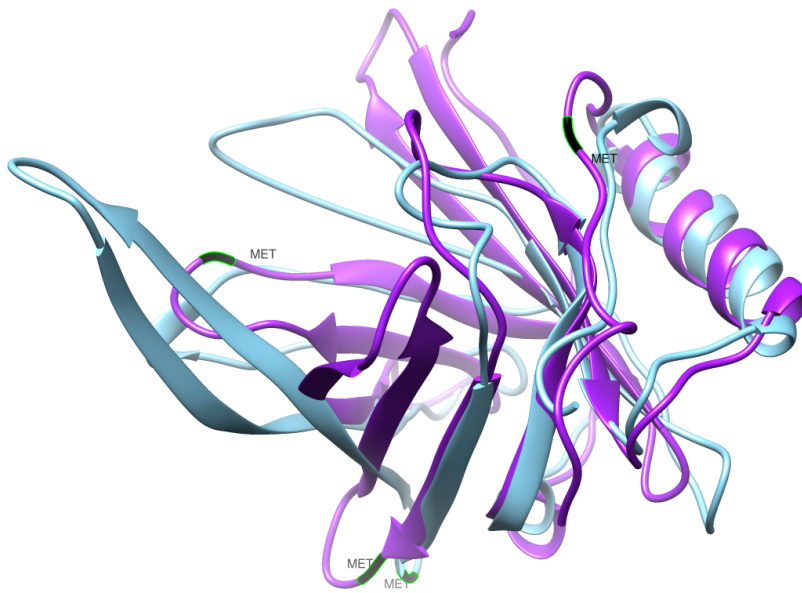


Figure 7



Supplementary Table 1

Supplementary Table 1. Sequences of PCR primers designed for the cloning of chimeric genes and transgenic plants characterization

TepGW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATATTATTAAGTAGTAGTATAAGC
TpepTil2	AACTAGGGAGTGCTGCTACAGAGAAGAAAGAGATGGAA
TilPep2	TTCCATCTCTTCTCTCTGTAGCAGCAGCACTCCCTAGTT
GWTiHA	GGGGACCACCTTTGTACAAGAAAGCTGGGTATTAAGCGTAATCTGGAACATCGTATGGGTATTTGCCGAAGAGAGATTTGAACCACC
Tpep long apoD	AACTAGGGAGTGCTGCTGCTCAAGCATTTCATCTTGGGAA
apoD Tpep long	TTCCCAAGATGAAATGCTTGAGCAGCAGCACTCCCTAGTT
GWapoD	GGGGACCACCTTTGTACAAGAAAGCTGGGTATTACGAGAGCTTGGGGCAGTTCAC